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Performance of *Clostridium difficile* Toxin Enzyme Immunoassay and Nucleic Acid Amplification Tests Stratified by Patient Disease Severity

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Many clinical laboratories in the United States are transitioning from toxin enzyme immunoassays (EIA) to nucleic acid amplification tests (NAATs) as the primary diagnostic test for *Clostridium difficile* infection (CDI). While it is known that the analytical sensitivity of the toxin EIA is poor, there are limited clinical data on the performance of these assays for patients with mild or severe CDI. Two hundred ninety-six hospital inpatients with diarrhea and clinical suspicion for CDI were tested prospectively by toxin EIA, by *C. difficile* NAAT, and with a reference standard toxigenic culture. Following completion of laboratory testing, retrospective chart reviews were performed to stratify patients into mild and severe disease groups based on clinical criteria using a standard point-based system. One hundred forty-three patients with CDI confirmed by toxigenic culture were evaluated in this study. Among the patients with mild CDI, 49% tested positive by toxin EIA and 98% tested positive by NAAT. Among patients with severe CDI, 58% tested positive by toxin EIA and 98% tested positive by NAAT. Increased CDI disease severity was not associated with an increased sensitivity of EIA ($P = 0.31$). These data demonstrate that toxin EIA performs poorly both for patients with severe CDI and for those with mild CDI and support the routine use of NAAT for the diagnosis of CDI. The presence of stool toxin measured by EIA does not correlate with disease severity.

Clostridium difficile infection (CDI) is a significant cause of morbidity and mortality in the health care setting. The incidence and severity of CDI are increasing in the United States (1), as is the number of patients who experience recurrent disease (2). Diagnosis of CDI requires evaluation of both clinical and laboratory findings. Patients may be considered to have CDI if they have both diarrhea (defined as passage of 3 or more unformed stools within a 24-h period) and a positive laboratory stool test for the presence of toxigenic *C. difficile* (2).

Laboratory tests available for the detection of *C. difficile* in stool specimens include culture, toxin antigen detection, and detection of toxin genes by nucleic acid amplification tests (NAATs). While culture for toxin-producing *C. difficile* is considered the gold standard, this test is ill suited to the clinical laboratory, as it is technically demanding and requires, at minimum, 3 days to perform. In contrast, enzyme immunoassays (EIA) for toxins A and/or B in stool have been widely used by clinical laboratories in the United States as a rapid method by which to detect *C. difficile.* However, the sensitivity of these EIAs is poor compared to culture, ranging from 33 to 65% (2, 3). In 2010, the Society for Healthcare Epidemiology (SHEA) and the Infectious Diseases Society of American (IDSA) indicated that toxin EIAs were no longer sufficient as standalone diagnostic tests for *C. difficile* (2). Because of their increased sensitivity and specificity compared to toxin EIAs (4–7), many laboratories are transitioning to NAATs as an alternative for the detection of *C. difficile.*

One main disadvantage of NAATs is that they do not detect the presence of biologically active toxin in stool specimens. The toxins expressed by *C. difficile* are this organism's main virulence factor, and some feel that the presence of toxin in stool is a positive correlate of disease (8). The significance of detecting *C. difficile* in the absence of the toxins, such as in the patient who tests positive by NAAT but negative by toxin EIA, is unclear. Furthermore, few well-controlled studies have established the clinical efficacy of NAATs (2), and none have evaluated the EIA and NAATs in parallel for the diagnosis of severe CDI. In this study, we investigated the sensitivity of a toxin A and B EIA and a *C. difficile* NAAT compared to toxigenic culture, stratified by CDI severity. Specifically, we sought to determine if patients who tested negative for *C. difficile* toxins by EIA but positive by NAAT were more likely to have mild CDI than patients who tested positive by both methods.

MATERIALS AND METHODS

Study population. The UCLA Health System (Los Angeles, CA) consists of a 300-bed acute care teaching hospital and a 600-bed tertiary care teaching hospital affiliated with the University of California, Los Angeles. From November 2011 through July 2012, adult inpatients were included in this study if they had a liquid stool specimen submitted to the clinical microbiology laboratory for *C. difficile* testing. All patients with a positive NAAT in the study were matched with an equal number of patients with negative NAAT results daily. All protocols were approved by the UCLA Institutional Review board.

Following completion of laboratory testing, retrospective chart reviews were performed in order to stratify patients into mild and severe disease groups based on the criteria of Zar and colleagues (9). Patients were assigned points based on age (>60 years, 1 point), temperature (>38.3°C, 1 point), albumin level (<2.5 mg/dl, 1 point), peripheral white blood cell (WBC) count (>15,000/mm^3, 1 point), treatment in the intensive care unite (ICU) (2 points), or endoscopic evidence of pseudomembranous colitis (2 points). Patients with ≥2 points were considered to have severe disease. Additional data on all patients were collected: hospital

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TABLE 1 Interpretation of laboratory test results for the diagnosis of CDI in patients with diarrhea and suspect *C. difficile* disease for this study[a]

Case definition	Laboratory test result				Interpretation	No. of patients (no. with severe disease)
	NAAT	EIA	Toxigenic culture	Cepheid Xpert		
CDI	−	−	+	−	NAAT and EIA FN	3 (1)
CDI	+	−	+	NP	EIA FN	51 (19)
CDI	−	+	+	NP	NAAT FN	0 (0)
CDI	+	+	+	NP	TP	70 (32)
CDI	+	−	−	+	EIA FN	13 (5)
CDI	+	+	−	+	Culture FN	6 (3)
No CDI	+	+	−	−	NAAT and EIA FP	0 (NA)
No CDI	+	−	−	−	NAAT FP	6 (NA)
No CDI	−	+	−	NP	EIA FP	9 (NA)
No CDI	−	−	−	NP	TN	138 (NA)

[a] EIA, toxin A and B enzyme immunoassay; FN, false negative; TP, true positive; FP, false positive; TN, true negatives; NP, not performed; NA, not applicable.

length of stay, 28-day all-cause mortality, laboratory evidence of recurrent *C. difficile* disease (e.g., positive NAAT on a liquid stool specimen submitted ≤1 month following appropriate treatment and abatement of symptoms), gastrointestinal disease comorbidity, immunosuppression, treatment with a stool softener, duration of symptoms, and number of stools on the day a specimen was collected for *C. difficile* testing. Community-associated CDI (CA-CDI) was defined as a positive *C. difficile* test within 3 days of hospital admission; all other cases were considered hospital-associated (HA)-CDI.

***C. difficile* testing.** Liquid stool specimens submitted to the microbiology laboratory were tested using the illumigene *C. difficile* (Meridian Bioscience, Cincinnati, OH) assay, a NAAT that detects the presence of *C. difficile* through amplification of the 5′ end of the toxin A gene. In parallel, a *C. difficile* toxin A/B immunoassay (Premier Toxin A+B; Meridian Bioscience) was performed on all samples. Samples were frozen at −20°C and shipped to a reference laboratory in batches for toxigenic culture by standard protocols. Results that were discrepant by toxigenic culture and NAAT were resolved by the Cepheid Xpert *C. difficile* assay, which detects the *tcdB* gene of *C. difficile*. Cases were defined as either patients with a positive toxigenic culture or patients who were toxigenic culture negative but tested positive by both the *C. difficile* illumigene and Cepheid Xpert tests (Table 1). Specimens that tested negative by EIA were further evaluated for potential postzone effect (e.g., antigen excess) by dilution of stool 1:10 and 1:100 in the Meridian Toxin A+B diluent and retesting in duplicate by the EIA, at these dilutions.

Statistical design and analysis. The study was powered using a 2-tailed β of 0.05 and α of 0.10, with the assumption of 90% sensitivity for the illumigene test to detect *C. difficile* and 60% sensitivity for the toxin A+B enzyme immunoassay. Based on these assumptions, a minimum of 40 patients with severe disease and 40 with mild disease were required in the study to detect a difference in the ability of the EIA and NAAT to diagnose patients with severe disease. At the time of study completion, 66 patients with severe disease and 89 with mild disease were included. Patient outcomes with respect to EIA results were compared using Fisher's exact test. Unpaired Student's *t* test and the Wilcoxon rank-sum test were used for comparison of continuous variables. A *P* value of <0.05 was considered statistically significant. All statistical analyses were performed using MedCalc Software version 12.3.0.0.

RESULTS

Patient characteristics. Two-hundred ninety-six patients with liquid stool sent for *C. difficile* testing were enrolled in the study. One hundred forty-three patients met the study criteria for laboratory-confirmed CDI (Table 1), whereas 153 patients tested negative for *C. difficile* (Table 1). Among the patients with CDI, 76 tested positive by both EIA and illumigene, 64 negative by EIA but positive by illumigene, and 3 negative by both EIA and illumigene (Table 1). Eighty-three cases (58%) were defined as mild disease and 60 (42%) as severe (Tables 1 and 2). Patients with mild disease were more likely to have CA-CDI (odds ratio [OR] = 1.51, $P = 0.05$) and shorter hospitalizations (average of 17 days versus 29 days for severe disease, $P = 0.002$) and had 2.7 times lower odds for all-cause mortality at 28 days ($P = 0.0001$; Table 2). No patients in this study had colectomy, and only one patient had endoscopic evidence of pseudomembranous colitis, though only 4 patients had endoscopy performed within 72 h of *C. difficile* testing. Ten patients died within 28 days of CDI diagnosis, all of whom were classified with severe disease.

Factors associated with false-negative toxin EIA results. Toxin EIA results were not correlated with CDI severity ($P = 0.31$; Table 3). Similarly, no difference was found between the EIA sensitivity levels in patients with severe (58% positive by EIA) and mild (49% positive by EIA) disease ($P = 0.31$, data not shown).

TABLE 2 Characteristics of patients with mild and severe CDI

Characteristic	Disease severity		OR	*P*[a]
	Mild ($n = 83$)	Severe ($n = 60$)		
No. (%) that were male	39 (46.9)	32 (51.6)		
No. (%) of patients with CA-CDI	38 (45.7)	18 (30)	1.51	0.05
No. (%) of patients with documented recurrent disease	14 (16.8)	10 (16.6)	0.98	1.0
Avg no. of stools per day ± SD	3 ± 3.4	3 ± 2.6		0.43
Avg duration of symptoms ± SD (days)	8 ± 3.1	6 ± 3.8		0.16
No. (%) of patients treated with immunosuppressive agent	25 (30.1)	10 (16.6)	0.67	0.09
No. (%) of patients treated with stool softener	34 (40.9)	22 (36.6)	0.99	1.0
No. (%) of patients with a GI[b] comorbidity	26 (31.3)	17 (28.3)	1.01	0.9
Mean hospital stay ± SD (days)	17 ± 20	29 ± 29		0.002
No. of patients with all-cause 1-mo mortality	0	10	2.66	0.0001

[a] *P* values were calculated using Fisher's exact test, except for average age, duration of symptoms, number of stools per day, and mean hospital days, which were calculated by the unpaired *t* test.
[b] GI, gastrointestinal.

TABLE 3 Characteristics of patients with CDI who tested positive or negative for *C. difficile* toxin A/B EIA

Characteristic	EIA result		OR	*P*[a]
	Negative	Positive		
Total no. of patients (male:female)	67 (31:36)	76 (40:36)		
Avg age ± SD	62.3 ± 20.6	65.8 ± 20.1		0.16
No. (%) >60 years of age	42 (63)	46 (61)	0.96	0.86
No. (%) under ICU treatment	11 (17)	18 (24)	1.22	0.30
No. (%) with temp of >38.3°C	8 (11.9)	8 (10.5)	0.93	0.79
No. (%) with albumin level of <2.5 mg/dl[b]	9 (28)	14 (35)	1.14	0.62
No. (%) with WBC count of >15,000 cells/mm^3	8 (11.9)	8 (10.5)	0.93	0.79
No. (%) showing presence of pseudomembranous colitis	0 (0)	1 (1.3)	1.89	1.00
No. (%) with severe disease	25 (37)	35 (46)	1.18	0.31
No. (%) with CA-CDI	31 (46)	27 (36)	1.18	0.31
No. (%) with documented recurrent disease	10 (16)	14 (18)	1.11	0.66
No. of stools per day ± SD	3 ± 3.6	3 ± 2.6		0.43
Duration of symptoms ± SD (days)	6 ± 6	8 ± 10.9		0.18
No. (%) of patients under treatment with immunosuppressive agent	24 (36)	24 (32)	0.91	0.71
No. (%) of patients with GI[c] comorbidity	22	21	0.62	0.31
Mean hospital stay ± SD (days)	18 (±18)	25 (±28)		0.04
No. (%) of patients with 1-mo all-cause mortality	5 (7.4)	5 (6.6)	1.01	1.00

[a] *P* values were calculated using Fisher's exact test, except for average age, duration of symptoms, number of stools per day, and mean hospital days, which were calculated by the unpaired *t* test.
[b] Only 72 patients were tested for albumin within 48 h of stool collection for *C. difficile* testing, encompassing 32 EIA-negative (48%) and 40 EIA-positive (53%) patients.
[c] GI, gastrointestinal.

Analysis of covariation for other patient characteristics measured in this study did not alter this result (not shown). The only factor identified by univariate analysis to be associated with EIA result was length of hospital stay (Table 3). Patients with positive EIAs had an average hospital stay of 25 ± 28 days, and patients with negative EIA results had an average hospital stay of 18 ± 18 days ($P = 0.04$; Table 3). Among the 10 patients who died within 28 days of CDI diagnosis, 5 tested positive by both EIA and NAAT and 5 tested negative by EIA and positive by NAAT (Table 3).

Performance of the illumigene *C. difficile* and toxin enzyme immunoassay for the detection of *C. difficile*. Sensitivity, specificity, and negative and positive predictive values for illumigene and toxin EIA are presented in Table 4. The sensitivity of the NAAT was 97.1% compared to toxigenic culture and 97.9% once culture/NAAT discrepant results were resolved by Cepheid Xpert testing. Three false-negative results were obtained by illumigene (Table 1); all three were culture positive but tested negative by both EIA and Cepheid Xpert. These patients included a 73-year-old man with mild disease and a history of antimicrobial exposure, a 31-year-old man with mild disease and no history of antimicrobial exposure, and a 54-year-old man with severe disease (defined in this patient by ICU treatment and WBC count of 15,600 cells/mm^3) and a history of antimicrobial exposure. Six false-positive results were obtained by illumigene, which tested negative by EIA, toxigenic culture, and Cepheid Xpert testing (Table 1).

In this study, EIA sensitivity was 47.1% compared to toxigenic culture and 53.1% compared to resolved NAAT results (data not shown). Resolved specificity was 94.1%; nine false-positive EIA results were documented in this study (Table 1). Specimens that were EIA negative but culture positive were diluted at 1:10 and 1:100 and retested in the attempt to identify a possible postzone effect; all diluted specimens remained EIA negative.

DISCUSSION

While no optimal strategy for clinical laboratory diagnosis of CDI has been defined, it is well recognized that the analytical sensitivity of the toxin EIA is unacceptably low (2). As toxin expression is thought to be one factor related to CDI severity (10), we sought to determine if false-negative toxin EIA results occurred in all patients with CDI or only in those patients with mild disease and presumably a low fecal concentration of *C. difficile* toxins (11). In contrast to this supposition, our data demonstrate that toxin EIA was negative in 42% of patients with severe CDI. NAAT, on the other hand, was negative in only one patient (2%) with severe disease, reinforcing the superior clinical performance of NAAT over EIA for laboratory diagnosis of CDI.

In this study, 60 patients were defined to have severe disease according to the criteria of Zar and colleagues (9). Factors associated with severe disease included HA-CDI and immunosuppression, and patients with severe disease were more likely to have longer hospitalizations and a higher rate of all-cause mortality at 28 days (Table 2). Alternative definitions for severe CDI have been proposed, including death within 30 days of *C. difficile* diagnosis, colectomy, or treatment in the ICU (12). No patients in our study

TABLE 4 Performance of illumigene *C. difficile* assay and toxin EIA compared to toxigenic culture for the detection of *C. difficile*

Characteristic[a]	illumigene results:				EIA results compared to:			
	Compared to toxigenic culture		Resolved by Cepheid testing		Toxigenic culture		Resolved illumigene results	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
Sensitivity	97.1	91.9–99.0	97.9	93.5–99.5	47.1	37.8–56.6	53.1	42.5–54.2
Specificity	79.8	71.7–86.1	96.1	80.2–92.2	87.4	91.3–98.4	94.1	88.8–97.1
PPV	80.8	72.6–87.1	95.9	90.9–98.3	76.6	64.0–85.8	89.4	80.4–94.7
NPV	96.9	90.7–99.2	98.0	93.8–99.4	65.4	57.4–72.6	68.2	61.4–74.3

[a] CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

required colectomy, but 29 patients required ICU treatment (11 of whom were EIA negative [Table 3]) and 10 patients died within 28 days of *C. difficile* diagnosis, including 5 EIA-negative patients (Table 3). Thus, even by more stringent criteria to define severe CDI, toxin EIA was not a reliable diagnostic test in our study.

Few other studies have evaluated toxin EIA performance in the context of patient disease severity, although de Jong and colleagues investigated peripheral white blood cell count in patients who tested negative or positive by toxin EIA. In their study, patients with toxin EIA-negative results were less likely to have leukocytosis of >15,000/ml than those patients who tested positive by both EIA and NAAT (13). However, only 10 patients were EIA negative/NAAT positive in this study. In contrast, among 64 patients with EIA/NAAT discordant results in our current study, no correlation between leukocytosis and EIA result was found (Table 3). In a large prospective study, Wilcox and colleagues recently demonstrated that the presence of toxin in stool specimens remains important to the definition of CDI. In their study, 6,524 hospital inpatients with diarrhea and suspected CDI were evaluated prospectively by both toxigenic culture and cell cytotoxicity neutralization assay (CCNA), which detects the presence of biologically active toxin through evaluation of cytopathic effect on Vero cells. CCNA results were found to correlate with 30-day mortality, length of hospitalization, and leukocytosis, whereas toxigenic culture did not correlate with these clinical characteristics (8; M. H. Wilcox, presented at ID Week 2012, San Diego, CA, 2012). These authors suggest that patients who test positive for *C. difficile* but negative for toxin be defined as "potential *C. difficile* excretors" for the purposes of infection control alone, but not necessarily be treated for CDI. However, in the Wilcox study, patients were not treated based on the CCNA results but rather on local *C. difficile* testing practices, and as such the outcomes associated with not treating patients with diarrhea and positive NAAT or glutamate dehydrogenase antigen (GDH) test but negative CCNA are unknown. Furthermore, how laboratories may test for the presence of toxin remains a dilemma, as in this same population, the sensitivity of the toxin EIAs was found to be only 68.2 to 82.3% compared to CCNA (14). Regardless, because CCNA may take up to 1 week to perform and requires laboratory proficiency in culturing cell monolayers and reading cytotoxicity, the United Kingdom's National Protection Agency endorses use of toxin EIAs to confirm the presence of toxin in patients who test positive for *C. difficile* by either NAAT or GDH screen. Outcome data from this approach, which is now in effect (http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_133016.pdf), will be revealing.

A clear etiology for EIA false-negative results has not yet been defined. Interstrain variability in toxin expression (15) may be one reason for false-negative toxin results (16). Alternatively, toxin may be diluted to below the limit of detection for the EIAs in some patients, due to increased volume and frequency of stools. However, in our analysis, no correlation was found between the number of stools per day and the EIA result (Table 3). Furthermore, repeat testing in two or more additional stool specimens by EIA does not result in an appreciable improvement in this test's sensitivity (17–19), suggesting that intermittent toxin shedding is not the basis for false-negative results. We evaluated specimens for postzone effect as a third possible cause of false-negative EIA results, but dilution and retesting of EIA-negative/NAAT-positive specimens did not yield any increase in EIA sensitivity. Finally, toxin stability may play a role in EIA results, although we did not evaluate specimen transport time to the laboratory in our study. The only factor associated with increased sensitivity of positive EIA results was greater length of hospitalization, although this correlation achieved a *P* value of relatively low significance (Table 3). Wilcox and colleagues noted that the presence of toxin also correlated with length of hospitalization when measured by CCNA (8). It is possible that these patients were infected with strains of *C. difficile* that express higher levels of toxin (10, 16) and thus required more lengthy hospitalizations, although we did not strain type the isolates in this study or evaluate their *in vitro* levels of toxin expression. Six false-positive results were obtained by the illumigene assay, an incidence similar to that noted by other studies (3, 6). The mechanism of these false positives is unclear, although some have suggested that loop-mediated isothermal amplification is more sensitive than toxigenic culture, and these may thus represent true-positive results, albeit at very low levels of organism presence (20, 21). However, others have reported that the sensitivity of the Xpert *C. difficile* assay, which was used as an arbiter test in our study, is greater than that of the illumigene assay (22).

While the clinical data to support routine use of NAAT for CDI diagnosis remain sparse, single institutional studies of patient outcomes before and after laboratory conversion from EIA to NAAT have demonstrated the benefits of NAAT. Benefits include fewer patients with CDI complications such as colectomy, admission to the ICU, and death (23) and earlier recognition of patients with CDI (24). NAATs may help with early diagnosis of CDI and may lead to treatment of patients before they progress to severe CDI, although this has not yet been specifically demonstrated. Additional benefits for the use of NAAT at an institutional level include elimination of unnecessary antimicrobial therapy for patients with presumptive, but toxin EIA-negative, CDI (25). It remains clear, however, that the use of laboratory stool tests in the diagnosis of CDI is complicated by those patients who are asymptomatically colonized with *C. difficile.* Use of colonoscopy, histopathology findings, and a sensitive toxin detection test, such as CCNA, may aid with diagnosis, but all laboratory tests must be interpreted in the context of patient symptoms and risk factors for CDI. As diarrhea is a common symptom in the hospitalized, elderly, or long-term care facility patient, it remains difficult to distinguish the patient with CDI from the patient for whom a positive *C. difficile* test is related to underlying colonization.

This study presents some limitations, primarily the fact that it was performed in a single center. Regardless, the findings strongly support the use of NAAT as the primary diagnostic laboratory test for CDI. While NAATs are roughly 10 times more costly than EIAs on a per test basis, prompt recognition of patients with CDI is imperative not only for patient management but also for infection prevention and control and antimicrobial stewardship.

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